Biosci. Biotechnol. Biochem., **70** (4), 949–957, 2006

Improved Production of Enzymes, Which Are Expressed under the Pho Regulon Promoter, in the *rmf* Gene (encoding ribosome modulation factor) Disruptant of *Escherichia coli*

Akira IMAIZUMI,† Chie KOSEKI, Kazuhiko MATSUI, and Hiroyuki KOJIMA

Laboratories of Fermentation and Biotechnology, AJINOMOTO Co., Inc., Kawasaki-shi, Kanagawa 210-8681, Japan

Received October 7, 2005; Accepted December 7, 2005

Using a DNA macroarray, we investigated the effects of *rmf* gene (encoding ribosome modulation factor) disruption on gene expression profiles in *Escherichia coli*. This strain showed a phosphate-starvation-like response in gene expression even under phosphate sufficient conditions; significant upregulation of the Pho regulon genes was observed. Further, the production of alkaline phosphatase, a product of the Pho regulon gene, *phoA*, increased in the *rmf* disruptant under a Pi sufficient condition. Furthermore, production of PhoC acid phosphatase/nucleoside pyrophosphate phosphotransferase derived from *Morganella morganii* also increased significantly in the *rmf* disruptant. We concluded that host modification by the *rmf* gene disruption has potential benefit in industrial enzyme production using *Escherichia coli*.

Key words: *Escherichia coli*; *Morganella morganii*; *rmf* gene; alkaline phosphatase; acid phosphatase/nucleoside pyrophosphate phosphotransferase

Microorganisms have developed various molecular mechanisms to survive in stressful environments. For example, in cells during the log growing phase, only the native form of ribosomes (70S) is observed. On the contrary, dimerized (100S) ribosomes that have no translation activity are observed in *Escherichia coli* when cells enter the stationary phase.[1] RMF (Ribosome Modulation Factor) protein has been detected as a factor related to this phenomenon.[2–5] RMF protein and its encoding gene *rmf* are expressed during the stationary phase, and RMF protein binds to 70S ribosomes to form the 100S ribosomes.[2–5] The 70S–100S interconversion of ribosomes might function as a mechanism controlling cellular protein synthesis during periods of starvation.[6–8]

Previously, we showed that disruption of the *rmf* gene increased the L-lysine productivity of the WC196 lysine-overproducing strain of *Escherichia coli* K-12,[9] demonstrating that inactivation of the *rmf* gene had merit for the industrial production of compounds potentially using *Escherichia coli*, but it is still unclear what happens in the *rmf* disruptant.

On the other hand, it was also reported that the effects of *rmf* gene disruption were only partial effects on recombinant protein production, suggesting that the rate of protein synthesis is dependent upon the promoter.[10] For example, no enhancement of protein production using *lac* promoter was observed, while significant enhancement was observed when acidic pH-inducible *cadA* promoter was used.[10] Therefore, to utilize *rmf* disruption in the development of industrial production using *Escherichia coli*, it is necessary to identify the genes whose expression levels are elevated in the disruptant.

In the present study, using gene expression profiling with a DNA-marcoarray, we attempted to identify the genes whose expression levels were elevated in the *rmf* disruptant of *E. coli* to exploit their promoters for the expression of various proteins. We found that Pho regulon genes, which were known as phosphate (Pi) starvation induced genes, were upregulated significantly in the *rmf* disruptant even under phosphate sufficient conditions. Furthermore, we attempted to express the exogenous protein in the *rmf* disruptant. The *phoC* gene encoding acid phosphatase derived from *Morganella morganii*[11] was expressed in the *rmf* disruptant of *E. coli*. Significantly higher production of the enzyme resulting from this was observed.

Materials and Methods

Strains, plasmids, and culture conditions. All the strains and plasmids used in this study are listed in Table 1. Cells were grown in L-broth containing 10 g/l Bacto Tryptone (Difco, Tokyo, Japan), 5 g/l yeast extract (Difco, Tokyo), 10 g/l NaCl, and modified MOPS minimal medium (MMM) containing 22.2 mM glucose, 50 mM NaCl, 0.523 mM NH_4Cl, 1 mM $(NH_4)_2SO_4$, 1 μM $FeSO_4 \cdot 7H_2O$, 5 μM $CaCl_2$, 10 μM $MnSO_4 \cdot 5H_2O$, 1 mM thiamine–HCl, 40 mM MOPS-KOH (pH

† To whom correspondence should be addressed. Tel: +81-44-210-5928; Fax: +81-44-244-4258; E-mail: akira_imaizumi@ajinomoto.com

Abbreviations: Pi, phosphate; MOPS, 3-morpholinopropanesulfonic acid; IPTG, isopropyl-beta-D-thiogalactopyranoside; OD, optical density

Table 1. Strains and Plasmids Used in This Study

Strain or plasmid	Genotype or gene	Source or reference
Strains		
MG1655	wild type	
MG1655R	MG1655Δ*rmf*	This study
WC196	Lysine producing mutant derived from W3110	Our laboratory stock
WC196R	WC196Δ*rmf*	9)
WC196R2	WC196Δ*rmf*::Cm$^{r a}$	This study
Plasmids		
pKD46	Helper plasmid expressing λ Red genes, Amp$^{r b}$	12)
pKD3	Template for amplification of Cm$^{r a}$ casstte, Cm$^{r a}$, Amp$^{r b}$	12)
pUC18	Plasmid vector, Amp$^{r b}$	Takara Bio
pMPI700	pUC18 carrying mutated *phoC* derived from *Morganella morganii*	16)
pMW118	Plasmid vector, Amp^{r}	Nippon Gene
pMW-phoA	pMW118 carrying wild type *phoA*	This study
pPRO LarA.122	Plasmid vector, Km$^{r c}$, IPTG + Arabinose inducible synthetic promoter	Clontech
pPRO LarA-phoA	pPRO LarA.122 carrying *phoA* gene under the control of IPTG + Arabinose inducible synthetic promoter	This study

[a] Chrolamphenicol-resistant.
[b] Ampicillin-resistant.
[c] Kanamycin-resistant.

7.2), and various concentrations of KH_2PO_4.

For growth assay, cells were collected from 50 μl of overnight culture grown in L-broth, then washed and inoculated into 5 ml of modified MMM in a TN-5L test tube (Advantec, Tokyo, Japan) at 37 °C aerobically. For gene expression profiling, 500 μl of the overnight culture was grown in L-broth, then washed and inoculated into 50 ml of modified MMM in a 500-ml shaking flask at 37 °C aerobically. For alkaline phosphatase assay and acid phosphatase production, cells were collected from 500 μl of the overnight culture grown in L-broth, then cultured 50 ml of L-broth in a 500-ml flask at 37 °C aerobically. For induction of β-galactosidase, 1 mM (final concentration) of IPTG (isopropyl-beta-D-thiogalactopyranoside) was added. For induction of pPRO LarA.-phoA, 1 mM (final concentration) of IPTG and 10 mM (final concentration) of L-Arabinose were added as required. In each culture, ampicillin (100 μg/ml) and kanamycin (25 μg/ml) were added as required.

DNA manipulations. For in-frame *rmf* gene disruption of MG1655, homologous recombination using plasmid pMANDR was performed as described previously (Table 1).[9] WC196R2 was constructed using the pharge λ Red disruption method developed by Datsenko and Wanner.[12] Plasmid pKD3 (Table 1) was used as a template and primers 5′-GTATGAGGGAAACGAGGCATGAAGAGACAAAAACGATGAAGCCTGCTTTTTTAT-3′ and 5′-GAATCAGGCCATTACTACCCTGTCCGCCATGGCTTCCGCTCAAGTTAGTATAAA-3′ were used. The resulting a 1.2 kb-fragment was introduced into WC196 harboring plasmid pKD46 (Table 1). The resulting WC196R2 carried a substitution of a 1.2 kb-fragment of the *rmf* gene to the *cat* gene (Table 1). The presence of the target mutations was confirmed by sequence analysis.

The 1.9 kb-fragment containing *phoA* open reading frame and promoter was amplified from MG1655 genomic DNA using primers 5′-ATTTTGTGTGGGTAATTATTTAAAT-3′ and 5′-GCGGATCCTTCACTGCCGGGCGCGGTTTTATTT-3′. The amplified fragment was digested with *Bam*HI and *Hin*dIII, and plasmid pMW118 (Takara Bio, Ohtsu, Japan) treated with *Bam*HI and *Hin*dIII was mixed and ligated, resulting in plasmid pMW-phoA (Table 1). The *phoA* gene was also cloned into the pPRO Lar.A122 (Clonetec, Mountain View, CA) plasmid vector which has IPTG and arabinose inducible promoter (Table 1). The 1.9 kb-fragment containing the *phoA* open reading frame was amplified from MG1655 genomic DNA using primers 5′-GCGCGGTACCCTTGTCACGGCCGAGACTTATAGTCGCT-3′ and 5′-GCGCAAGCTTTTGTGTTGCTTGATTATTGCATTCG-3′, then the amplified fragment was digested with *Kpn*I and *Hin*dIII, and plasmid pPRO Lar.A122 treated with *Kpn*I and *Hin*dIII was mixed and ligated (Table 1). All other basic recombinant DNA procedures, such as isolation and purification of DNA, restriction enzyme digestion, and transformation of *E. coli*, were performed as described by Sambrook *et al.*[13]

DNA macroarrays and data analysis. Total RNA was isolated in the growth phase before Pi starvation when the OD_{660} reached 0.3 (t = approx. 1.5 h), and after the cells entered Pi starvation when the OD_{600} reached 0.5 (t = approx. 4 h), as described previously.[9] Random hexamer-primed ^{33}P-labeled cDNA synthesis was carried out as described previously.[9]

Hybridization and quantification of each spot were performed as described previously.[9] The spot density of each gene was normalized as the ratio to the total density of all genes per membrane. The normalized data (two

duplicates per culture) were analyzed using a regularized *t*-test based on a Bayesian statistical framework,[14,15] which was utilized to calculate *p*-values for comparison of mRNA expression between the experimental and control groups. The analysis was implemented using the Cyber-T website (http://visitor.ics.uci.edu/genex/cybert/). A *p*-value of less than 0.001 was considered statistically significant.

Measurement and analysis. For flask cultivation, the optical density (OD) was measured using a Beckman DU-640 spectrometer. For growth assay, the OD was measured automatically with a TN-1506 incubator (Advantec, Tokyo).

For alkaline phosphatase assay, the periplasmic fraction was extracted as described previously[16] and the residual Pi was removed using a PD-10 column (Amersham, Tokyo, Japan). The phosphatase activity was determined according to the generation of *p*-nitrophenolphosphate by measuring absorbance at 410 nm in a reaction mixture containing 100 mM Tris–HCl (pH 8.0), 1 mM $MgCl_2$, 0.05 mM $ZnCl_2$, and 10 mM *p*-nitrophenylphosphate at 37 °C, using bacterial alkaline phosphatase (Takara Bio) as a standard. The reaction was stopped by the addition of an equivalent volume of 1 M K_2HPO_4.

For acid phosphatase assay, crude extract was extracted using Bug-Buster (Novagen, Darmstadt, Germany) according to the manufacturer's protocol. Activities were determined according to the generation of *p*-nitrophenolphosphate by measuring absorbance at 410 nm in a reaction mixture containing 100 μM of morpholine ethanesulfonic acid (MES)–NaOH buffer (pH 6.0), and 10 mM *p*-nitrophenylphosphate as described previously.[17]

β-galactosidase activites were determined at 30 min after addition of IPTG, and measured as described previously.[18]

For the measurement of extracellar Pi concentration, supernatant of culture was collected and then measured using the method described previously.[19]

SDS–polyacrylamide gel electrophoresis (SDS–PAGE). Crude extract containing 30 μg of protein was separated by SDS–PAGE, as described by Laemmli,[20] with a gradient gel consisting of 4 to 20% of acrylamide (Bio-Rad, Tokyo, Japan). SDS–PAGE standard (Bio-Rad) was used as the protein standard. The gel was stained with Sypro Orange (Bio-Rad), then the image was scanned using an FLA-3000 fluorescent image analyszer (Fuji Film, Tokyo, Japan).

Results

Growth assay of the rmf disruptant under Pi-limited conditions

In our previous study, the *rmf* disruptant of an L-lysine overproducing strain, WC196R, showed higher growth than the parental strain under phosphate-limited conditions.[9] This result suggests that disruption of the *rmf* gene alters the response to external Pi concentration. MG1655 and its *rmf*-disruptant MG1655R were cultivated in modified MMM supplemented with 500, 50, or 5 μM of KH_2PO_4 as sole phosphorus source. Immediately after entering the stationary phase, glucose levels, not Pi levels, were depleted when the cells were cultivated with 500 μM KH_2PO_4, whereas Pi levels were depleted when the cells were cultivated with 50 or 5 μM KH_2PO_4 (data not shown). The two strains showed similar growth curves when they were cultivated with sufficient Pi (500 μM), but MG1655R showed higher growth under Pi-limited conditions (50 or 5 μM) (Fig. 1A). Thus the observation in our previous study[9] was reproduced in MG1655, the wild-type strain of *E. coli* K-12.

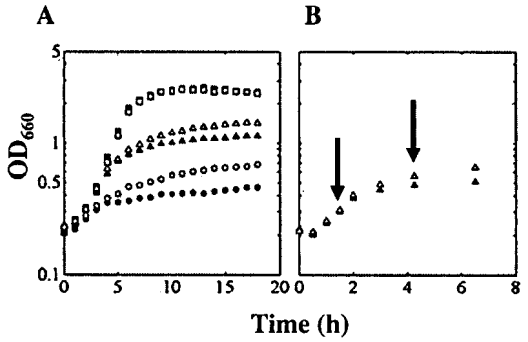

Fig. 1. Effects of Phosphate Limitation and *rmf* Disruption on Growth.

A, Growth curves of MG1655 (closed symbols) and MG1655R (open symbols). Cells were cultivated in TN-1506 incubator (ADVANTEC, Tokyo, Japan) with modified MMM containing 500, 50, or 5 μM of KH_2PO_4 (shown as squares, triangles, and circles respectively). The experiment was performed three times independently, resulting in almost the same cultivation profiles. B, Growth curve of MG1655 (closed symbols) and MG1655R (open symbols). Cells were cultivated in a 500-ml shaking flask with 50 ml of modified MMM containing 50 μM KH_2PO_4. Arrows represent the points at which the gene expression was profiled.

Gene-expression profiles of MG1655 and MG1655R under Pi-sufficient and Pi-starved conditions

Next the gene-expression profiles of MG1655 and MG1655R under Pi-sufficient and Pi-starved conditions were investigated using DNA macroarrays. Cells were grown in modified MMM supplement with 50 μM KH_2PO_4 in a 500-ml shaking flask, and total RNA was extracted at 1.5 h and 4 h (Fig. 1B). At 1.5 h, it was confirmed that Pi was not yet depleted in the culture of either strain (data not shown).

Interestingly, the correlation coefficient for the gene-expression levels of the MG1655 and MG1655R at 1.5 h was quite low: the correlation coefficient was as low as 0.389. On the other hand, the gene expression profiles of MG1655R at 1.5 h and 4 h, and that of MG1655 at 4 h

Table 2. Genes Up- or Down-Regulated Significantly in MG1655R under Pi-Sufficient Conditions

Down-regulated genes

Gene	*p* value[a]	Fold change[b]	Gene product[c]
rplA	1.54E-06	−4.49	50S ribosomal subunit protein L1
pyrB	2.09E-06	−4.23	aspartate carbomoyltransferase catalytic subunit
b1875	2.65E-04	−3.94	f190; This 190 aa ORF is 38 pct identical (3 gaps) to 127 residues of an approx. 144 aa protein YF82 HAEIN SW: P44262
yebL	3.16E-08	−3.88	31.1 kD protein in msbB-ruvB intergenic region
rplL	8.37E-09	−3.63	50S ribosomal subunit protein L7/L12
rpsF	1.50E-06	−3.58	30S ribosomal subunit protein S6
b0296	1.04E-07	−3.54	f87; 100 pct identical to GB: ECU73857 Accession U73857; residues 21–77 are 38 pct identical to aa 8-64 from 50S ribosomal protein L31, RL31 HAEIN SW: P44367 (70 aa)
rplV	2.70E-07	−3.46	50S ribosomal subunit protein L22
rplU	7.88E-09	−3.45	50S ribosomal subunit protein L21
rplK	4.83E-05	−3.42	50S ribosomal subunit protein L11
rplP	3.09E-05	−3.39	50S ribosomal subunit protein L16
rpsS	9.70E-09	−3.38	30S ribosomal subunit protein S19
yceD	7.48E-04	−3.30	hypothetical 19.3 kD protein in rne-rpmF intergenic region
sodA	5.96E-05	−3.29	manganese superoxide dismutase
rplC	1.51E-07	−3.20	50S ribosomal subunit protein L3
rpmE	8.18E-04	−3.18	50S ribosomal protein L31
carA	1.93E-05	−3.15	carbamoyl-phosphate synthase small chain
rpmC	6.80E-07	−3.15	50S ribosomal subunit protein L29
rplQ	1.72E-06	−3.12	50S ribosomal subunit protein L17
rpmA	1.00E-05	−3.12	50S ribosomal subunit protein L27
pyrI	6.64E-09	−3.10	aspartate carbomoyltransferase regulatory subunit
rplD	4.42E-06	−3.08	50S ribosomal subunit protein L4
rplS	1.19E-05	−3.06	50S ribosomal subunit protein L19
glnA	2.00E-07	−3.05	glutamine synthetase
rpsC	4.12E-06	−3.01	30S ribosomal subunit protein S3
rpoA	1.43E-11	−3.00	f329; CG Site No. 234; 100 pct identical amino acid sequence and equal length to RPOA ECOLI SW: P00574
rpmF	9.80E-04	−2.96	50S ribosomal protein L32
purC	3.60E-06	−2.96	phosphoribosylaminoimidazole-succinocarboxamide synthase
rpsR	2.82E-04	−2.95	30S ribosomal subunit protein S18
rpsT	1.58E-06	−2.94	30S ribosomal protein S20

Continued on next page.

were quite similar: the correlation coefficients were about 0.893–0.954. Additionally, a significant change in gene expression profile was also observed in MG1655 between 1.5 h and 4 h: the correlation coefficient was 0.366.

Next, to confirm that the significant differencein gene expression observed at 1.5 h was not to be attributed to Pi availability, the gene expression profiles of the two strains grown under high-Pi level (500 μM) were analyzed. The gene expression profile grown under high-Pi level at 1.5 h was quite similar to that at 1.5 h under low-Pi level in the two strains (data not shown). Therefore, these results demonstrate that the gene expression profile of MG1655R was highly similar to the Pi-starvation response observed in MG1655, regardless of Pi availability.

Then, using a regularized *t*-test based on a Bayesian statistical framework, statistical analysis between the gene expression profiles of the two strains at 1.5 h was performed.[14,15] Genes up- or down-regulated in MG1655R significantly ($p < 0.001$) are listed in Table 2. Several ribosomal protein coding genes (19 of the 30 most down-regulated genes) were downregulated significantly (Table 2). Additionally, the *rpoA* gene, coding the alpha-subunit of the RNA polymerase core enzyme, was also downregulated significantly (Table 2). Among the upregulated genes, several genes known as Pho regulon genes, whose transcription is known to be induced under Pi-starved conditions, were detected in MG1655R, although external Pi was not yet depleted (Table 2). For example, the *phoA* gene, coding alkaline phosphatase, was one of the most upregulated genes (Table 2). Other Pho regulon genes, *pstS*, *phnB*, *phoE*, *phnF*, and *phoB*, were also upregulated significantly (Table 2). Expression of Pho regulon genes is controlled by the PhoBR two-component system, which is composed of PhoR histidine kinase/phosphatase and the PhoB response regulator.[21] The kinase activity of PhoR is activated when extracellular phosphate levels are low (< 4 μM); under these conditions, PhoB is phosphorylated and activated by PhoR, which results in enhancement of Pho regulon genes transcription.[21,22] It is known that all Pho regulons are preceded by a promoter containing an upstream binding site, the Pho

Table 2. (continued)

Up-regulated genes

Gene	*p* value[a]	Fold change[b]	Gene product[c]
yibD	1.08E-04	**5.56**	hypothetical 40.5 kD protein in secB-tdh 5′ region
xasA	2.64E-05	**4.84**	XasA
phoA	1.98E-05	**4.77**	alkaline phosphatase precursor
b2080	7.33E-04	**4.68**	o123
b0753	1.92E-05	**4.29**	f126
pstS	4.36E-05	**4.15**	periplasmic phosphate-binding protein
gadE	3.85E-10	**4.10**	hypothetical 20.6 kD protein in hdeD-gadA intergenic region
gadA	2.97E-07	**4.06**	GAD alpha protein
phnB	5.53E-06	**4.03**	phnB protein
phoE	2.02E-04	**3.88**	outer membrane pore protein E precursor
b4045	7.79E-05	**3.72**	o69
tktB	5.03E-04	**3.60**	transketolase 2
yiaG	4.15E-07	**3.58**	hypothetical 11.0 kD protein in bisC-cspA intergenic region
b2028	4.75E-07	**3.49**	f388; This 388 aa ORF is 93 pct identical (16 gaps) to 388 residues of an approx. 376 aa protein UDG SHIFL SW: P37791
yjbA	1.31E-06	**3.44**	o136
phoU	1.26E-05	**3.33**	peripheral membrane protein U
hdeB	6.80E-05	**3.19**	protein hdeB precursor
ygjG	1.99E-04	**3.08**	probable ornithine aminotransferase
pflB	4.90E-04	**3.04**	formate acetyltransferase 1
wrbA	8.94E-07	**3.00**	trp repressor binding protein
gltB	5.17E-07	**2.97**	glutamate synthase (NADPH) large chain precursor
b1967	1.37E-06	**2.94**	o283; This 283 aa ORF is 23 pct identical (18 gaps) to 232 residues of an approx. 248 aa protein YA02 SCHPO SW: Q09675
b1810	3.51E-06	**2.93**	o119; This 119 aa ORF is 38 pct identical (2 gaps) to 39 residues of an approx. 424 aa protein GA83 YEAST SW: Q04739
phnF	4.12E-07	**2.92**	phnf protein
hdeA	1.67E-09	**2.92**	protein hdeA precursor
b2266	2.65E-05	**2.91**	f101; 41 pct identical amino acid sequence and equal length to YQJD ECOLI SW: P42617
b0329	2.31E-06	**2.87**	o91; 33 pct identical (2 gaps) to 86 residues of approx. 112 aa protein YHCN ECOLI SW: P46477
b2255	8.89E-09	**2.86**	o660; This 660 aa ORF is 30 pct identical (9 gaps) to 301 residues of an approx. 320 aa protein FMT ECOLI SW: P23882
b1843	9.48E-04	**2.81**	o218; This 218 aa ORF is 24 pct identical (2 gaps) to 87 residues of an approx. 2056 aa protein FAS1 YEAST SW: P07149
phoB	2.40E-05	**2.81**	phosphate regulon transcriptional regulatory protein PhoB

[a]The *p* value was calculated as described in the literature[14] (see "Materials and Methods").
[b]Fold change was determined as $\log_2$ of the averaged expression levels of MG1655R per MG1655.
[c]The annotation is sited by information generously provided by http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?val=U00096

box sequence, for phosphorylated PhoB protein.[21] It been thought that Pho regulon genes were upregulated only when extracellar Pi levels are low.[21,23] But, our results indicate that this system might also work when extracellar Pi levels are higher than the threshold against an *rmf*-disrupted background. Based on these results, we focused on the *phoA* gene, one of the Pho regulon genes, for further analysis.

Increased expression of alkaline phosphatase in the rmf disruptant

The effects of the *rmf* gene disruption on alkaline phosphatase activities were investigated. A *phoA*-expressing plasmid, pMW-phoA, was constructed (see "Materials and Methods") and the alkaline phosphatase activities of MG1655, MG1655R and those strains harboring pMW-phoA were measured. After growth in L-broth for 6 h, alkaline phosphatase assay was performed. Regardless of the copy number of the *phoA* gene, an approximately 1.3 fold increase in alkaline phosphatase activities was observed in the *rmf* disruptants (Table 3). It was also confirmed that Pi was not yet exhausted completely in each culture (data not shown). Therefore, the increase of alkaline phosphatase observed in MG1655R was not to be attributed to Pi-starvation.

Then the effect of promoter sequence was investigated using the *phoA* gene under the control of IPTG and the arabinose inducible promoter. The *phoA* gene was cloned into the pPRO Lar.A122 plasmid vector and introduced into MG1655 and MG1655R. Although a remarkable (4 fold) enhancement of activities with the addition of IPTG and arabinose was observed, there were no significant difference in activities between control and the *rmf* gene disruptant (Table 3). Therefore, the increase in alkaline phosphatase activity observed in the *rmf*-disrupted strains might be attributed to upregu-

Table 3. Effects of *rmf* Gene Disruption and Promoters on Alkaline Phosphatase Activities

Strain	Plasmid	IPTG, Arabinose	Activity ± SD[a] (U/mg-protein)	Ratio[b]
MG1655			0.065 ± 0.012	
MG1655R			0.080 ± 0.012	**1.23**
WC196			0.039 ± 0.004	
WC196R			0.055 ± 0.016	**1.39**
WC196R2			0.049 ± 0.005	**1.24**
MG1655	pMW-phoA		0.134 ± 0.010	
MG1655R	pMW-phoA		0.169 ± 0.022	**1.27**
MG1655	pPRO LarA-phoA		0.118 ± 0.009	
MG1655R	pPRO LarA-phoA		0.122 ± 0.013	**1.03**
MG1655	pPRO LarA-phoA	added	0.444 ± 0.053	
MG1655R	pPRO LarA-phoA	added	0.455 ± 0.003	**1.02**

[a]Standard deviations of three replicates.
[b]The ratio was determined as the average enzymatic activity of the *rmf* disruptant per that of the wild type.

lation of transcription through the *phoA* promoter in which the Pho box sequence was located.

To confirm that the increase in alkaline phosphatase activity was to be attributed neither to the polar effect caused by *rmf* gene disruption nor to the genetic background, the alkaline phosphatase activities of the lysine overproducing strain WC196[9] and their various types of *rmf* gene disruptant, WC196R (in-frame *rmf* gene disruption) and WC196R2 (the *rmf* gene was substituted for the *cat* gene) were measured. Regardless of the features of the disruptant, almost the same extent of increase in enzymatic activity was observed in each of the disruptants (Table 3). These observations suggest that the upregulation of the *phoA* gene was not dependent on the genetic background and not to be attributed to the polar effect.

Enhancement of PhoC acid phosphatase production in the rmf disruptants

Plasmid pMPI700 carries an 1.2-kb DNA fragment of mutant *phoC* gene derived from *Morganella morganii*.[11,17] The gene product also catalyzes a nucleoside phosphorylation reaction, as shown in the following equation: nucleoside + pyrophosphate → nucleoside 5′-monophosphate + P_i.[16] The products of this reaction, for example inosine 5′-monophosphate (5′-IMP) and guanosine 5′-monophosphate (5′-GMP), are widely used as flavor potentiators in various foods. By harboring plasmid pMPI700 in which the *phoC* gene was expressed under its own promoter, 5′-IMP was produced at a practical level.[17] Therefore, an increase in the expression level of this enzyme has an impact on the industrial production process.

A nucleotide sequence homologous to known Pho box[21] sequences was identified within the 1.2-kb DNA fragment (Fig. 2). This sequence was located near the predicted promoter region of the *phoC* gene (Fig. 2). It is known that the Pho box is located upstream of the −10 region of the promoter,[21] suggesting that the promoter enhanced by PhoB was the second of the predicted promoter sequences (the −35 region was located from −92 to −86, and the −10 region was located from −80 to −76) (Fig. 2). Hence the effect of *rmf* gene disruption on enzyme production of PhoC acid phosphatase was investigated by means of harboring plasmid pMPI700. After growth in L-broth for 6 h, acid phosphatase activities were measured. A significant increase in acid phosphatase activity was observed in the *rmf* disruptant (Table 4). To confirm that the enhanced level of enzymatic activity corresponded to the increase in enzyme production, crude extracts obtained from WC196 harboring pMPI700 and WC196R harboring pMPI700 were analyzed by SDS gel electrophoresis (Fig. 3A). The density of the corresponding band (approx. 25 kDa) in the *rmf* disruptant was significantly greater than in the parental strain, and the level of enhancement of protein expression corresponded to that of the enzyme activity (Fig. 3A). Hence the growth and enzymatic activity of WC196 harboring pMPI700 and WC196R harboring pMPI700 were monitored periodi-

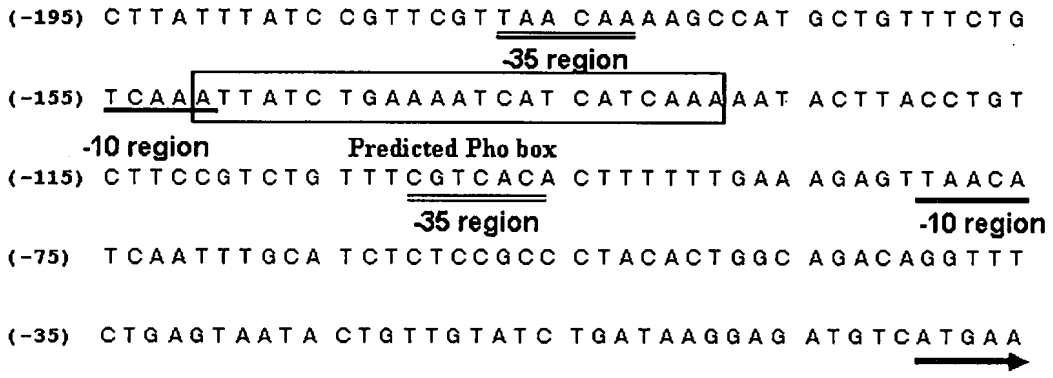

Fig. 2. DNA Sequence of *phoC* Upstream Region of Plasmid pMPI700.
The predicted Pho box sequence is indicated by the box. Predicted −35 regions and −10 regions of σ^{70} dependent promoter are doubly and singly underlined respectively. The translation starting position is indicated by an arrow.

Table 4. Effects of *rmf* Gene Disruption on the Enzymatic Activities of Acid Phosphatase Derived from *Morganella morganii*
Plasmid pMPI700 was introduced into each strain.

Strain	Activity ± SD[a] (U/mg-protein)	Ratio[b]
MG1655	4.56 ± 0.17	1.49
MG1655R	6.79 ± 0.82	
WC196	2.02 ± 0.39	2.02
WC196R	4.08 ± 0.47	

[a]Standard deviations of three replicates.
[b]The ratio was determined as the average enzymatic activity of the *rmf* disruptant per that of wild type.

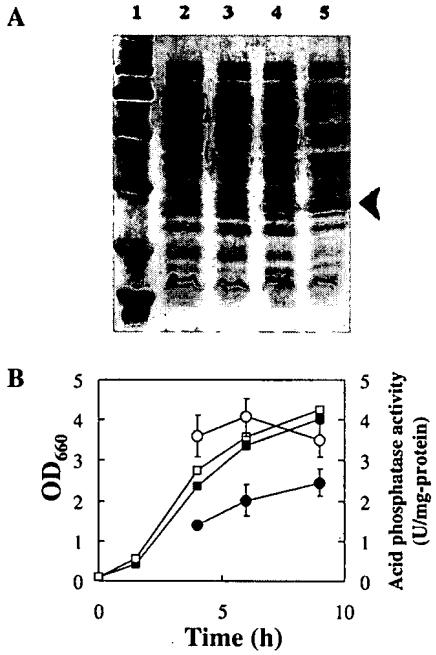

Fig. 3. Expression of PhoC Acid Phosphatase Derived from *Morganella morganii* in *Escherichia coli*.
A, SDS–PAGE analysis of crude extract of *E. coli* WC196 (lane 2), WC196R (lane 3), WC196 transformed with pMPI700 (lane 4), and WC196R transformed with pMPI700 (lane 5). Lane 1, Protein markers (from the top, 94, 67, 43, 30, 21, 14.4, and 6.5 kDa). PhoC acid phosphatase is indicated by the arrowhead. B, Growth (squares) and acid phosphatase activities (circles) of WC196/pMPI700 (solid symbols) and WC196R/pMPI700 (open symbols). Error bars indicate the standard deviations of three replicates.

cally. At every point, a significant increase in acid phosphatase activities was observed in the disruptant (Fig. 3B), but no delay of growth was observed in it (Fig. 3B).

Discussion

In this study, higher growth was observed in the *rmf*-inactivated strain under Pi-starved conditions, confirming the previous study (Fig. 1). According to gene expression profiling, the gene expression profiles of MG1655R were similar to those of MG1655 under Pi-starved conditions regardless of extracellar Pi-levels (Table 2). To put it concretely, it was found that expression of Pho regulon genes considered to be upregulated only under Pi-starved conditions until now[23] were also upregulated significantly even under the Pi-enriched environment in the *rmf* gene-inactivated background (Table 2). According to our results, the gene expression profile changed dramatically in the *rmf* disruptant even in the growing phase (Fig. 1B). This result suggests that the *rmf* gene was expressed in the wild-type strain under the growing phase. It is known that the *rmf* gene is expressed even in the growing phase under slow-growth conditions.[2] It has also been reported that expression of the *rmf* gene requires intracellar accumulation of ppGpp (guanosine 3′, 5′-tetraphosphate).[24] ppGpp is a pleiotropic regulator that controls the expression of many genes in response to various stresses such as amino acid starvation.[25] In this experiment, cells were grown under an amino acid-free condition (see "Materials and Methods"). Hence, it was suggested that the phenomenon observed in this experiment is to be attributed to the intracellar accumulation of ppGpp due to amino acid starvation.

Increases in the enzymatic activities of PhoA alkaline phosphatase and PhoC acid phosphatase derived from *Morganella morganii* were observed both in the *rmf* disruptant of MG1655 and in that of WC196, when they were expressed under the control of promoters containing Pho box sequences in a Pi-sufficient environment (Table 3, Table 4, and Fig. 3). Moreover, it was also confirmed that no significant increase in *lac* promoter-controlled β-galactosidase activities occured in the *rmf* disruptants when plasmid pUC18, the vector plasmid used for the construction of pMPI700, was introduced (data not shown), confirming the previous study.[10] Therefore, the increase in enzyme production observed in the *rmf* disruptant might be attributed to the characteristics of the promoter and not to the copy number of the plasmid. According to the known function of RMF protein, these phenomena are not to be attributed to the direct effect of the *rmf* disruption but to secondary effects. One possible hypothesis is that the difference in growth rate between wild-type strain and the *rmf* disruptant under a Pi-limited environment is due to a change in phosphate metabolism, although the mechanisms are still unclear. In any case, host modification by the *rmf* gene disruption has potential benefit in industrial enzyme production using *Escherichia coli*.

On the other hand, a significant downregulation of ribosomal protein coding genes and related genes was observed in MG1655R even when the cells were growing at the same growth rate as that of the wild-type strain, MG1655 (Table 2). In general, the number of ribosomes per cell is in proportion to the growth rate,[26,27] and ribosome synthesis is regulated by strict feedback repression that maintains the number of

ribosomes, in what is known as "growth rate-dependent control."[28–30] RMF protein is considered to participate in this process.[6–8] In the *rmf*-disruptant strain, the ribosomes might appear to remain in the monomeric active form.[2,9] Therefore, in the *rmf*-disruptant strain, it should be possible to maintain the number of ribosomes per cell adequately by means of some other way besides dimerization of ribosomes. Moreover, the transcription of ribosomal proteins is thought to be synchronized with that of ribosomal RNA.[31–33] Taking account of this, the downregulation of ribosomal protein coding genes observed in MG1655R might be attributed to the strict feedback repression of ribosome synthesis resulting from disruption of the *rmf* gene. The synthesis of ribosomes requires large amounts of energy and nucleosides, phosphates, and other substrates. It has been suggested that the RMF protein might play an important role, in conjunction with feedback repression, in regulating the number of active ribosomes in *E. coli*.[30] Further studies on the relationships among the regulation of active ribosomes, phosphate metabolisms, and growth are in progress.

Acknowledgments

We thank Dr. Yasuhiro Mihara for providing experimental materials, and Dr. Akira Wada, Dr. Kan Tanaka, and Dr. Yoshihiro Usuda for valuable discussions and suggestions. We also thank all of our colleagues at the Fermentation and Biotechnology Laboratories of Ajinomoto Co., Inc., Japan.

References

1) Wada, A., Yamazaki, Y., Fujita, N., and Ishihama, A., Structure and probable genetic location of a "ribosome modulation factor" associated with 100S ribosomes in stationary-phase *Escherichia coli* cells. *Proc. Natl. Acad. Sci. USA*, **87**, 2657–2661 (1990).
2) Yamagishi, M., Matsushima, H., Wada, A., Sakagami, M., Fujita, N., and Ishihama, A., Regulation of the *Escherichia coli rmf* gene encoding the ribosome modulation factor: growth phase- and growth rate-dependent control. *EMBO J.*, **12**, 625–630 (1993).
3) Wada, A., Igarashi, K., Yoshimura, S., Aimoto, S., and Ishihama, A., Ribosome modulation factor: stationary growth phase-specific inhibitor of ribosome functions from *Escherichia coli*. *Biochem. Biophys. Res. Commun.*, **214**, 410–417 (1995).
4) Wada, A., Growth phase coupled modulation of *Escherichia coli* ribosomes. *Genes to Cells*, **3**, 203–208 (1998).
5) Ishihama, A., Modulation of the nucleoid, the transcription apparatus, and the translation machinery in bacteria for stationary phase survival. *Genes to Cells*, **4**, 135–143 (1999).
6) Yoshida, H., Maki, Y., Kato, H., Fujisawa, H., Izutsu, K., Wada, C., and Wada, A., The ribosome modulation factor (RMF) binding site on the 100S ribosome of *Escherichia coli*. *J. Biochem.* (Tokyo), **132**, 983–989 (2002).
7) El-Sharoud, W. M., Ribosome disruption for preservation: concepts and reservations. *Sci. Prog.*, **87**, 137–152 (2004).
8) Yoshida, H., Yamamoto, H., Uchiumi, T., and Wada, A., RMF inactivates ribosomes by covering the peptidyl transferase center and entrance of peptide exit tunnel. *Genes to Cells*, **9**, 271–278 (2004).
9) Imaizumi, A., Takikawa, R., Koseki, C., Usuda, Y., Yasueda, H., Kojima, H., Matsui, K., and Sugimoto, S., Improved production of L-lysine by disruption of stationary phase-specific *rmf* gene in *Escherichia coli*. *J. Biotechnol.*, **117**, 111–118 (2005).
10) Chou, C. H., Bennett, G. N., and San, K. Y., Genetic manipulation of stationary-phase genes to enhance recombinant protein production in *Escherichia coli*. *Biotechnol. Bioeng.*, **50**, 636–642 (1996).
11) Thaller, M. C., Berlutti, F., Schippa, S., Lombardi, G., and Rossolini, G. M., Characterization and sequence of PhoC, the principal phosphate-irrepressible acid phosphatase of *Morganella morganii*. *Microbiology*, **140**, 1341–1350 (1994).
12) Datsenko, K. A., and Wanner, B. L., One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. USA*, **97**, 6640–6645 (2000).
13) Sambrook, J., Fritsch, E. F., and Maniatis, T., Molecular cloning: a laboratory manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor (1989).
14) Baldi, P., and Long, A. D., A Bayesian framework for the analysis of microarray expression data: regularized *t*-test and statistical inferences of gene changes. *Bioinformatics*, **17**, 509–519 (2001).
15) Hung, S., Baldi, P., and Hatfield, G. W., Global gene expression profiling in *Escherichia coli* K12: the effects of leucine responsive regulatory protein. *J. Biol. Chem.*, **277**, 40309–40323 (2002).
16) Kaderbhai, M. A., Ugochukwu, C. C., Kelly, S. L., and Lamb, D. C., Export of cytochrome P450 105D1 to the periplasmic space of *Escherichia coli*. *Appl. Environ. Microbiol.*, **67**, 2136–2138 (2001).
17) Mihara, Y., Utagawa, T., Yamada, H., and Asano, Y., Phosphorylation of nucleosides by the mutated acid phosphatase from *Morganella morganii*. *Appl. Environ. Microbiol.*, **66**, 2811–2816 (2000).
18) Miller, J. H., "Experiments in Molecular Genetics," Cold Spring Harbor Laboratory Press, Cold Spring Harbor (1972).
19) Fiske, C. H., and Subbarow, Y., The colorimetric determination of phosphorus. *J. Biol. Chem.*, **66**, 375–400 (1925).
20) Laemmli, U. K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685 (1970).
21) Wanner, B. L., Phosphorus assimilation and control of the phosphate regulon. In "*Escherichia coli* and *Salmonella*: Cellular and Molecular Biology" 2nd edition, ed. Neidhardt, F. C., ASM Press, Washington, DC, pp. 1357–1381 (1996).
22) Makino, K., Amemura, M., Kim, S. K., Nakata, A., and Shinagawa, H., Regulation of the phosphate regulon of *Escherichia coli*: activation of *pstS* transcription by PhoB protein *in vitro*. *J. Mol. Biol.*, **203**, 85–95 (1988).

23) Pasternak, N. T., Yagil, E., and Spira, B., A differential effect of σ^S on the expression of the PHO regulon gene in *Escherichia coli*. *Microbiology*, **100**, 2985–2992 (2004).
24) Izutsu, K., Wada, A., and Wada, C., Expression of ribosome modulation factor (RMF) in *Escherichia coli* requires ppGpp. *Genes to Cells*, **6**, 665–676 (2001).
25) Cashel, M., Gentry, D. R., Hernadez, V. J., and Vinella, D., The stringent response. In "*Escherichia coli* and *Salmonella*: Cellular and Molecular Biology" 2nd edition, ed. Neidhardt, F. C., ASM Press, Washington, DC, pp. 1458–1496 (1996).
26) Bremer, H., and Dennis, P., Modulation of chemical composition and other parameters of the cell by growth rate. In "*Escherichia coli* and *Salmonella*: Cellular and Molecular biology" 2nd edition, ed. Neidhardt, F. C., ASM Press, Washington, DC, pp. 1553–1569 (1996).
27) Maaløe, O., An analysis of bacterial growth. *Dev. Biol. Suppl.*, **3**, 33–58 (1969).
28) Gourse, R. L., Gaal, T., Bartlett, M. S., Appleman, J. A., and Ross, W., rRNA transcription and growth rate-dependent regulation of ribosome synthesis in *Escherichia coli*. *Annu. Rev. Microbiol.*, **50**, 645–677 (1996).
29) Condon, C., Liveris, D., Squires, C. I., Schwartz, I., and Squires, C. L., rRNA operon multiplicity in *Escherichia coli* and the physiological implications of *rrn* disruption. *J. Bacteriol.*, **177**, 4152–4156 (1995).
30) Keener, J., and Nomura, M., Regulation of ribosome synthesis. In "*Escherichia coli* and *Salmonella*: Cellular and Molecular Biology" 2nd edition, ed. Neidhardt, F. C., ASM Press, Washington, DC, pp. 1417–1431 (1996).
31) Dean, D., and Nomura, M., Feedback regulation of ribosomal protein gene expression in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA*, **77**, 3590–3594 (1980).
32) Nomura, M., Yates, J. L., Dean, D., and Post, L. E., Feedback regulation of ribosomal protein gene expression in *Escherichia coli*: structural homology of ribosomal RNA and ribosomal protein mRNA. *Proc. Natl. Acad. Sci. USA*, **77**, 7084–7088 (1980).
33) Nomura, M., Gourse, R., and Baughman, G., Regulation of the synthesis of ribosomes and ribosomal components. *Annu. Rev. Biochem.*, **53**, 75–117 (1984).